

JC890 U.S. PTO  
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PATENT

Attorney Docket No. DAVI103.001AUS

Date: August 2, 2000

Page 1

ASSISTANT COMMISSIONER FOR PATENTS

WASHINGTON, D.C. 20231

ATTENTION: BOX PATENT APPLICATION

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): **Geoffrey Charles Nicholson**

For: **A METHOD OF TREATMENT AND AGENTS USEFUL FOR SAME**

Enclosed are:

- (X) 6 sheets of drawing.
- (X) Sequence Listing in 6 pages.
- (X) Return prepaid postcard.

JC836 U.S. PTO  
09/632074  
08/02/00

CLAIMS AS FILED

| FOR                                      | NUMBER<br>FILED | NUMBER<br>EXTRA | RATE  | FEE   |
|--|-----------------|-----------------|-------|-------|
| Basic Fee                                |                 |                 | \$690 | \$690 |
| Total Claims                             | 17 - 20 =       | ×               | \$18  | \$0   |
| Independent Claims                       | 4 - 3 =         | 1 ×             | \$78  | \$78  |
| FILING FEE TO BE PAID<br>AT A LATER DATE |                 | \$768           |       |       |

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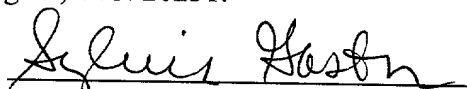
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Attorney Docket No. : DAVI103.001AUS  
Applicant(s) : Nicholson  
For : A METHOD OF TREATMENT AND  
AGENTS USEFUL FOR SAME  
Attorney : Daniel E. Altman  
"Express Mail"  
Mailing Label No. : EL559437695  
Date of Deposit : August 2, 2000

I hereby certify that the accompanying

Transmittal in Duplicate; Specification in 27 pages; 6 sheets of drawings;  
Sequence Listing in 6 pages; Return Prepaid Postcard

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## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

|           |   |                       |   |                        |
|-----------|---|-----------------------|---|------------------------|
| Applicant | : | Nicholson, G.         | ) | Group Art Unit Unknown |
|           |   |                       | ) |                        |
| Appl. No. | : | Unknown               | ) |                        |
|           |   |                       | ) |                        |
| Filed     | : | Herewith              | ) |                        |
|           |   |                       | ) |                        |
| For       | : | A METHOD OF TREATMENT | ) |                        |
|           |   | AND AGENTS USEFUL FOR | ) |                        |
|           |   | SAME                  | ) |                        |
|           |   |                       | ) |                        |
| Examiner  | : | Unknown               | ) |                        |

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Preliminary to Examination on the merits, please amend the above-captioned application as follows:

**IN THE SPECIFICATION:**

On page 1 of the Specification, line 2, after the Title of the Invention, please insert --This Application claims priority under 35 U.S.C. §119(a) of Australian Provisional Patent application No. PQ1999, filed August 3, 1999.--.

On page 24 of the Specification, before Claim 1, please cancel the word "CLAIMS" and substitute in its place --WHAT IS CLAIMED IS:--.

**IN THE CLAIMS:**

**Please cancel Claims 11 and 12.**

**Please amend the remaining claims as follows:**

1. (Amended) A method of modulating bone resorption in an [human or] animal, said method comprising administering to said [human or] animal an effective amount of a leptin

Appl. No. : Unknown  
Filed : Herewith

or a derivative, homologue, analogue, chemical equivalent, antagonist or agonist thereof for a time and under conditions sufficient for the modulation of osteoclastogenesis.

2. (Amended) [A]The method according to Claim 1 wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in [~~400~~7]SEQ ID NO:7 after optimal alignment.

3. (Amended) [A]The method according to Claim 1, wherein the leptin or its derivative, homologue, antagonist or agonist is encoded by the nucleotide sequence set forth in [~~400~~8]SEQ ID NO:8 or a nucleotide sequence having at least 60% similarity to [~~400~~8]SEQ ID NO:8 after optimal alignment or a nucleotide sequence capable of hybridizing to [~~400~~8]SEQ ID NO:8 or its complementary from under low stringency conditions at 42°C.

4. (Amended) [A]The method according to Claim 1 [or 2 or 3] wherein the modulation comprises a reduction in bone resorption.

5. (Amended) [A]The method according to Claim 4 [for the treatment]wherein said bone resorption is a result of osteoporosis or Paget's disease.

6. (Amended) A method for inhibiting, reducing or otherwise delaying onset or progression of bone resorption in an [human or] animal, said method comprising administering to said [human or] animal an effective amount of a leptin or a derivative, homologue, analogue, chemical equivalent or agonist thereof for a time and under conditions sufficient to inhibit, reduce or otherwise delay onset or progression of osteoclastogenesis.

7. (Amended) [A]The method according to Claim 6, wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in [~~400~~7]SEQ ID NO:7 after optimal alignment.

8. (Amended) [A]The method according to Claim 7 wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence have at least 60% similarity to the amino acid sequence set forth in [~~400~~7]SEQ ID NO:7 after optimal alignment.

9. (Amended) [A]The method according to Claim 6 [or 7 or 8 for the treatment]wherein said bone resorption is a result of osteoporosis or Paget's disease.

Appl. No. : Unknown  
Filed : Herewith

10. (Amended) A composition for modulating bone resorption, comprising a leptin [or a derivative, homologue, analogue, chemical equivalent, antagonist or agonist thereof]having at least 60% similarity to the amino acid sequence set forth in SEQ ID NO:7 and one or more pharmaceutically acceptable carriers and/or diluents [**when used for modulating bone resorption**].

13. (Amended) A method for inhibiting osteoclastogenesis in an [human or] animal, said method comprising administering to said [human or] animal an amount of a leptin or a derivative, homologue, analogue, chemical equivalent or agonist thereof effective to antagonize the osteoclastic effect of osteoclast differentiation factor (ODF) by stimulation of Osteoprotegerin (OPG) and/or inhibition of receptor activator of NF-kappa  $\beta$  (RANK) expression.

14. (Amended) [A]The method according to Claim 13, wherein the leptin or its derivatives, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in [~~400~~>7]SEQ ID NO:7 after optimal alignment.

15. (Amended) [A]The method according to Claim 13 wherein the leptin or its derivative, homologue, antagonist or agonist is encoded by the nucleotide sequence set forth in [~~400~~>8]SEQ ID NO:8 or a nucleotide sequence having at least 60% similarity to [~~400~~>8]SEQ ID NO:8 after optimal alignment or a nucleotide sequence capable of hybridizing to [~~400~~>8]SEQ ID NO:8 or its complementary form under low stringency conditions at 42°C.

16. (Amended) [A]The method according to Claim 13 [**or 14 or 15 for the treatment of**]wherein said bone resorption is a result osteoporosis or Paget's disease.

**Please add the following claims:**

17. The method of Claim 1 wherein said animal is a human.
18. The method of Claim 6 wherein said animal is a human.
19. The method of Claim 13 wherein said animal is a human.

#### **REMARKS**

The claims have been amended to conform to the rules of practice specified by the U.S. Patent and Trademark Office. Claims 11 and 12 have been canceled. Claims 17-19 have been added. No new matter has been added herewith. As a result of this Preliminary Amendment, Claims 1-10, and 13-19 are presented for examination.

Appl. No. : Unknown  
Filed : Herewith

**Conclusion**

Should there be any questions concerning this application, the Examiner is respectfully requested to contact the undersigned attorney at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 2 Aug. 2000

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## A METHOD OF TREATMENT AND AGENTS USEFUL FOR SAME

### FIELD OF THE INVENTION

5 The present invention relates generally to a method of modulating bone resorption and to agents useful for same. More particularly, the present invention provides for the use of leptin and its derivatives, homologues, analogues, antagonists or agonists to modulate osteoclastogenesis. Even more particularly, the present invention contemplates the treatment of disorders characterised by or associated with excessive bone resorption such as but not  
10 limited to osteoporosis and Paget's disease. The present invention further provides for the use of leptin and its derivatives, homologues, analogues, antagonists and agonists in the manufacture of a medicament for the modulation of bone resorption.

### BACKGROUND OF THE INVENTION

15 Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Nucleotide and amino acid sequences are referred to by a sequence identifier, i.e. <400>1, <400>2, etc. A sequence listing is provided after the claims.

20 Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other country.

25 The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine,  
30 H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

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The regulation of bone metabolism is a multifaceted process requiring the tight control of bone resorption and bone formation. The latter is the primary function of osteoblasts whereas bone resorption involves osteoclasts.

5

Osteoclasts are multinucleate cells formed in bone marrow by the fusion of cells from the monocyte/macrophage lineage (Suda *et al.*, 1992; Quinn *et al.*, 1998). A variety of factors play a role in regulating osteoclast formation including growth factors, systemic hormones and cell contact with marrow stroma.

10

A number of proteins have been identified which are involved in the process of osteoclastogenesis. Osteoprotegerin, also known as osteoclastogenesis inhibitory factor (OPG and OCIF, respectively), is a secreted member of the TNF receptor superfamily that blocks osteoclast differentiation both *in vitro* and *in vivo* (Yasuda, *et al.*, 1998; Simonet *et al.*, 1997). The cloning of a membrane bound ligand for OPG (OPG-ligand [OPGL]) resulted in the identification of an essential signal for proliferation and fusion of osteoclast progenitors (Yasuda, *et al.*, 1998). This protein, also called osteoclast differentiation factor (ODF), is expressed on the plasma membrane of osteoblasts/marrow stromal cells and has a membrane bound receptor (in contrast to the soluble receptor, OPG/OCIF) identified as receptor activator of NF-kappa  $\beta$  (RANK). OPGL/ODF has also been termed TNF-related activation-induced cytokine (TRANCE) and RANK-ligand (RANKL). The combination of M-CSF and a soluble form of recombinant ODF, lacking the transmembrane domain, is necessary and sufficient to stimulate osteoclast generation, in the absence of osteoblast or stromal cells, from either murine spleen cells or human monocytes (Matsuzaki *et al.*, 1998; Quinn *et al.*, 1988).

25

Leptin, a cytokine produced primarily by mature adipocytes, is linked to food intake and energy expenditure (Friedman and Halaas, 1998) but also has activity in neuroendocrine, metabolic, reproductive and haemopoetic pathways (Auwerz and Staels, 1998).

30



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In work leading up to the present invention, the inventors investigated the role of leptin in the bone microenvironment. The inventors have now identified leptin as a regulator of osteoclastogenesis. This provides the basis for the development of a range of medicaments for modulating bone resorption.

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## SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of  
5 a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

One aspect of the present invention contemplates a method of modulating bone resorption in a human or animal, said method comprising administering to said human or animal an  
10 effective amount of leptin or a derivative, homologue, analogue, chemical equivalent, antagonist or agonist thereof for a time and under conditions sufficient for the modulation of osteoclastogenesis.

Another aspect of the present invention provides a method for inhibiting, reducing or  
15 otherwise delaying onset or progression of bone resorption in a human or animal, said method comprising administering to said human or animal an effective amount of a leptin as hereinbefore defined for a time and under conditions sufficient to inhibit, reduce or otherwise delay onset or progression of osteoclastogenesis.

Yet another aspect of the present invention is directed to the use of leptin as hereinbefore  
20 defined in the manufacture of a medicament in the treatment of a disease condition involving excess bone resorption.

Still yet another aspect of the present invention provides a composition useful in the  
25 modulation of bone resorption comprising leptin as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents.

- 5 -

**BRIEF DESCRIPTION OF THE FIGURES**

**Figure 1** is a photographic representation showing generation of TRAP +ve multinuclear cells (MNC) and monoclear (Mono) cells. A colour version of this figure is available by request from the Applicant.

**Figure 2** is a graphical representation showing the effect of leptin on the generation of TRAP +ve multinuclear (MNCs). TRAP +ve MNCs were generated on bone slices from PBMCs treated with ODF (55 ng/ml) and M-CSF (25 ng/ml) for 21 days. a, b, c denote significant difference,  $p > 0.05$ , ANOVA, Fischer's multiple comparison.

**Figure 3** is a graphical representation showing the effect of leptin on bone resorption by ODF/M-CSF generated human osteoclasts. The percentage of bone surface resorbed by human osteoclast generated from PBMCs was quantified by SEM. a, b, c denotes significant difference,  $p > 0.05$ , ANOVA, Fischer's multiple comparison.

**Figure 4** shows photographic and graphical representations of the effects of leptin on OPG and RANK mRNA expression. Adherent PBMCs were treated with leptin for 24 hr immediately after settlement. The expression of OPG, RANK and GAPDH mRNA in human PBMCs was quantified using semi-quantitative RT-PCR (A). The net intensity of OPG and RANK product bands of mRNA expression were analysed and corrected for GAPDH (B).

**Figure 5** is a graphical representation of the effect of leptin on osteoclastogenesis in purified CD14+ cells. CD14+ cells were positively selected from unfractionated PBMCs using anti-CD14 antibody labelled immunomagnetic beads and cultured on bone slices for 3 weeks in the presence of M-CSF (25 ng/ml) and ODF (40 ng/ml) with and without leptin 1.6 Fg/ml.

**Figure 6** is a schematic representation of the proposed mechanism of leptin inhibition of osteoclastogenesis. Not shown on the figure is the likely production of leptin by bone marrow adipocytes.

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Abbreviations used in the subject specification are defined in Table 1.

TABLE 1

| ABBREVIATIONS |  |
|---------------|--|
| TNF           | Tumour necrosis factor   |
| OPG (OCIF)    | Osteoprotegerin; Osteoclastogenesis Inhibitory Factor  |
| OPGL (ODF)    | Membrane bound ligand for OPG; osteoclast differentiation factor (same as TRANCE, TNF-related activation-induced cytokine) |
| RANKL         | Ligand for RANK (same as OPGL/ODF/TRANCE)  |
| RANK          | Receptor activator of NP-kappa $\beta$   |
| M-CSF         | Macrophage-colony stimulating factor   |
| Mono          | Mononuclear cell   |
| MNC           | Multinuclear cell  |
| TRAP          | Tartrate-resistant acid phosphatase  |
| PBMC          | Peripheral blood mononuclear cell  |
| PBS           | Phosphate buffered saline  |
| FCS           | Fetal calf serum   |
| TGF $\beta$   | Transforming growth factor - $\beta$   |
| RT-PCR        | Reverse transcriptase polymerase chain reaction  |
| CTR           | Calcitonin receptor  |

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## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification of leptin as a potent inhibitor of osteoclastogenesis. This provides a means for modulating bone resorption.

5

Accordingly, one aspect of the present invention contemplates a method of modulating bone resorption in a human or animal, said method comprising administering to said human or animal an effective amount of leptin or a derivative, homologue, analogue, chemical equivalent, antagonist or agonist thereof for a time and under conditions sufficient for the modulation of osteoclastogenesis.

10

Reference herein to "leptin" includes reference to a polypeptide having the amino acid sequence set forth in <400>7 or an amino acid sequence having at least 60% similarity thereto while retaining leptin activity or antagonist activity as well as a molecule encoded by the nucleotide sequence set forth in <400>8 or a nucleotide sequence having at least about 60% similarity thereto or a nucleotide sequence capable of hybridising to <400>8 under low stringency conditions at 42°C.

15

Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridisation, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out  $T_m = 69.3 + 0.41 (G+C)\%$  (Marmur and Doty, 1962). However, the  $T_m$  of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, 1974).

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The term "leptin" is defined herein as including all derivatives, homologues, analogues, chemical equivalents, antagonists and agonists thereof.

- 5 The term "derivative" and its plural form includes parts, portions, fragments, regions, fusion molecules, mimotopes and mimetics.

10 Analogues and mimetics include molecules which contain non-naturally occurring amino acids as well as molecules which do not contain amino acids but nevertheless behave functionally the same as leptin. Natural product screening is one useful strategy for identifying analogues and mimetics. Natural product screening involves screening environments such as bacteria, plants, animals, rainforests, riverbeds, seabeds, aquatic environments, coral and antarctic or arctic environments for naturally occurring molecules which mimic, agonize or antagonize leptin of the present invention. Analogues of leptin  
15 contemplated herein include modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptide molecule or their analogues.

- 20 Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with  $\text{NaBH}_4$ ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups  
25 with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with  $\text{NaBH}_4$ .

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

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The carboxyl group may be modified by carbodimide activation *via* O-acylisourea formation followed by subsequent derivitization, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 2.

- 10 -

TABLE 2

| Non-conventional amino acid               | Code  | Non-conventional amino acid       | Code   |
|---|-------|-----------------------------------|--------|
| $\alpha$ -aminobutyric acid               | Abu   | L-N-methylalanine                 | Nmala  |
| $\alpha$ -amino- $\alpha$ -methylbutyrate | Mgab  | L-N-methylarginine                | Nmarg  |
| aminocyclopropane-                        | Cpro  | L-N-methylasparagine              | Nmasn  |
| carboxylate                               |       | L-N-methylaspartic acid           | Nmasp  |
| aminoisobutyric acid                      | Aib   | L-N-methylcysteine                | Nmcys  |
| aminonorbomyl-                            | Norb  | L-N-methylglutamine               | Nmgln  |
| carboxylate                               |       | L-N-methylglutamic acid           | Nmglu  |
| cyclohexylalanine                         | Chexa | L-N-methylhistidine               | Nmhis  |
| cyclopentylalanine                        | Cpen  | L-N-methylisoleucine              | Nmile  |
| D-alanine                                 | Dal   | L-N-methylleucine                 | Nmleu  |
| D-arginine                                | Darg  | L-N-methyllysine                  | Nmlys  |
| D-aspartic acid                           | Dasp  | L-N-methylmethionine              | Nmmet  |
| D-cysteine                                | Dcys  | L-N-methylnorleucine              | Nmnle  |
| D-glutamine                               | Dgln  | L-N-methylnorvaline               | Nmnva  |
| D-glutamic acid                           | Dglu  | L-N-methylornithine               | Nmorn  |
| D-histidine                               | Dhis  | L-N-methylphenylalanine           | Nmphe  |
| D-isoleucine                              | Dile  | L-N-methylproline                 | Nmpro  |
| D-leucine                                 | Dleu  | L-N-methylserine                  | Nmser  |
| D-lysine                                  | Dlys  | L-N-methylthreonine               | Nmthr  |
| D-methionine                              | Dmet  | L-N-methyltryptophan              | Nmtrp  |
| D-ornithine                               | Dorn  | L-N-methyltyrosine                | Nmtyr  |
| D-phenylalanine                           | Dphe  | L-N-methylvaline                  | Nmval  |
| D-proline                                 | Dpro  | L-N-methylethylglycine            | Nmetg  |
| D-serine                                  | Dser  | L-N-methyl-t-butylglycine         | Nmtbug |
| D-threonine                               | Dthr  | L-norleucine                      | Nle    |
| D-tryptophan                              | Dtrp  | L-norvaline                       | Nva    |
| D-tyrosine                                | Dtyr  | $\alpha$ -methyl-aminoisobutyrate | Maib   |

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|                                  |        |   |        |
|----------------------------------|--------|---|--------|
| D-valine                         | Dval   | $\alpha$ -methyl- $\gamma$ -aminobutyrate   | Mgab   |
| D- $\alpha$ -methylalanine       | Dmala  | $\alpha$ -methylcyclohexylalanine           | Mchexa |
| D- $\alpha$ -methylarginine      | Dmarg  | $\alpha$ -methylcyclopentylalanine          | Mcpen  |
| D- $\alpha$ -methylasparagine    | Dmasn  | $\alpha$ -methyl- $\alpha$ -naphthylalanine | Manap  |
| D- $\alpha$ -methylaspartate     | Dmasp  | $\alpha$ -methylpenicillamine               | Mpen   |
| D- $\alpha$ -methylcysteine      | Dmcys  | N-(4-aminobutyl)glycine                     | Nglu   |
| D- $\alpha$ -methylglutamine     | Dmgln  | N-(2-aminoethyl)glycine                     | Naeg   |
| D- $\alpha$ -methylhistidine     | Dmhis  | N-(3-aminopropyl)glycine                    | Nom    |
| D- $\alpha$ -methylisoleucine    | Dmile  | N-amino- $\alpha$ -methylbutyrate           | Nmaabu |
| D- $\alpha$ -methylleucine       | Dmleu  | $\alpha$ -naphthylalanine                   | Anap   |
| D- $\alpha$ -methyllysine        | Dmlys  | N-benzylglycine                             | Nphe   |
| D- $\alpha$ -methylmethionine    | Dmmet  | N-(2-carbamylethyl)glycine                  | Ngln   |
| D- $\alpha$ -methylornithine     | Dmorn  | N-(carbamylmethyl)glycine                   | Nasn   |
| D- $\alpha$ -methylphenylalanine | Dmphe  | N-(2-carboxyethyl)glycine                   | Nglu   |
| D- $\alpha$ -methylproline       | Dmpro  | N-(carboxymethyl)glycine                    | Nasp   |
| D- $\alpha$ -methylserine        | Dmser  | N-cyclobutylglycine                         | Ncbut  |
| D- $\alpha$ -methylthreonine     | Dmthr  | N-cycloheptylglycine                        | Nchep  |
| D- $\alpha$ -methyltryptophan    | Dmtrp  | N-cyclohexylglycine                         | Nchex  |
| D- $\alpha$ -methyltyrosine      | Dmtty  | N-cyclodecylglycine                         | Ncdec  |
| D- $\alpha$ -methylvaline        | Dmval  | N-cyclododecylglycine                       | Ncdod  |
| D-N-methylalanine                | Dnmala | N-cyclooctylglycine                         | Ncoct  |
| D-N-methylarginine               | Dnmarg | N-cyclopropylglycine                        | Ncpro  |
| D-N-methylasparagine             | Dnmasn | N-cycloundecylglycine                       | Ncund  |
| D-N-methylaspartate              | Dnmasp | N-(2,2-diphenylethyl)glycine                | Nbhm   |
| D-N-methylcysteine               | Dnmcys | N-(3,3-diphenylpropyl)glycine               | Nbhe   |
| D-N-methylglutamine              | Dnmgln | N-(3-guanidinopropyl)glycine                | Narg   |
| D-N-methylglutamate              | Dnmglu | N-(1-hydroxyethyl)glycine                   | Nthr   |
| D-N-methylhistidine              | Dnmhis | N-(hydroxyethyl)glycine                     | Nser   |
| D-N-methylisoleucine             | Dnmile | N-(imidazolylethyl)glycine                  | Nhis   |
| D-N-methylleucine                | Dnmleu | N-(3-indolylethyl)glycine                   | Nhtrp  |
| D-N-methyllysine                 | Dnmlys | N-methyl- $\gamma$ -aminobutyrate           | Nmgabu |

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|                                  |         |   |        |
|----------------------------------|---------|---|--------|
| N-methylcyclohexylalanine        | Nmchexa | D-N-methylmethionine                        | Dnmmt  |
| D-N-methylornithine              | Dnmorn  | N-methylcyclopentylalanine                  | Nmcpn  |
| N-methylglycine                  | Nala    | D-N-methylphenylalanine                     | Dnmphe |
| N-methylaminoisobutyrate         | Nmaib   | D-N-methylproline                           | Dnmpro |
| N-(1-methylpropyl)glycine        | Nile    | D-N-methylserine                            | Dnmser |
| N-(2-methylpropyl)glycine        | Nleu    | D-N-methylthreonine                         | Dnmthr |
| D-N-methyltryptophan             | Dnmtrp  | N-(1-methylethyl)glycine                    | Nval   |
| D-N-methyltyrosine               | Dnmtyr  | N-methyl- $\alpha$ -naphthylalanine         | Nmanap |
| D-N-methylvaline                 | Dnmval  | N-methylpenicillamine                       | Nmpen  |
| $\gamma$ -aminobutyric acid      | Gabu    | N-( <i>p</i> -hydroxyphenyl)glycine         | Nhtyr  |
| L- <i>t</i> -butylglycine        | Tbug    | N-(thiomethyl)glycine                       | Ncys   |
| L-ethylglycine                   | Etg     | penicillamine                               | Pen    |
| L-homophenylalanine              | Hphe    | L- $\alpha$ -methylalanine                  | Mala   |
| L- $\alpha$ -methylarginine      | Marg    | L- $\alpha$ -methylasparagine               | Masn   |
| L- $\alpha$ -methylaspartate     | Masp    | L- $\alpha$ -methyl- <i>t</i> -butylglycine | Mtbug  |
| L- $\alpha$ -methylcysteine      | Mcys    | L-methylethylglycine                        | Metg   |
| L- $\alpha$ -methylglutamine     | Mgln    | L- $\alpha$ -methylglutamate                | Mglu   |
| L- $\alpha$ -methylhistidine     | Mhis    | L- $\alpha$ -methylhomophenylalanine        | Mhphe  |
| L- $\alpha$ -methylisoleucine    | Mile    | N-(2-methylthioethyl)glycine                | Nmet   |
| L- $\alpha$ -methylleucine       | Mleu    | L- $\alpha$ -methyllysine                   | Mlys   |
| L- $\alpha$ -methylmethionine    | Mmet    | L- $\alpha$ -methylnorleucine               | Mnle   |
| L- $\alpha$ -methylnorvaline     | Mnva    | L- $\alpha$ -methylornithine                | Morn   |
| L- $\alpha$ -methylphenylalanine | Mphe    | L- $\alpha$ -methylproline                  | Mpro   |
| L- $\alpha$ -methylserine        | Mser    | L- $\alpha$ -methylthreonine                | Mthr   |
| L- $\alpha$ -methyltryptophan    | Mtrp    | L- $\alpha$ -methyltyrosine                 | Mtyr   |
| L- $\alpha$ -methylvaline        | Mval    | L-N-methylhomophenylalanine                 | Nmhphe |
| N-(N-(2,2-diphenylethyl)         | Nnbhm   | N-(N-(3,3-diphenylpropyl)                   | Nnbhe  |
| carbamylmethyl)glycine           |         | carbamylmethyl)glycine                      |        |
| 1-carboxy-1-(2,2-diphenyl-       | Nmbc    |   |        |
| ethylamino)cyclopropane          |         |   |        |

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Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having  $(CH_2)_n$  spacer groups with  $n=1$  to  $n=6$ , glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of  $C_\alpha$  and  $N_\alpha$ -methylamino acids, introduction of double bonds between  $C_\alpha$  and  $C_\beta$  atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

All these types of modifications may be important to stabilize leptin. This may be important if leptin is used, for example, in the manufacture of a therapeutic composition.

The present invention further contemplates chemical equivalents of leptin. Chemical equivalents may not necessarily be derived from leptin itself but may share certain conformational or functional similarities. Alternatively, chemical equivalents may be specifically designed to mimic certain physiochemical properties of the polypeptides. Chemical equivalents may be chemically synthesized or may be detected following, for example, natural product screening.

The term "modulate" means that bone resorption may be stimulated, enhanced or otherwise increased or that it may be inhibited, retarded or otherwise reduced. Reduction in bone resorption is important for disease conditions involving an excess of bone resorption such as osteoporosis or Paget's disease. Preferably, the modulation involves a reduction in bone resorption.

Accordingly, another aspect of the present invention provides a method for inhibiting, reducing or otherwise delaying onset or progression of bone resorption in a human or animal, said method comprising administering to said human or animal an effective

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amount of a leptin as hereinbefore defined for a time and under conditions sufficient to inhibit, reduce or otherwise delay onset or progression of osteoclastogenesis.

Yet another aspect of the present invention is directed to the use of leptin as hereinbefore defined in the manufacture of a medicament in the treatment of a disease condition involving excess bone resorption.

Such conditions include osteoporosis and Paget's disease.

Accordingly, the present invention provides a composition useful in the modulation of bone resorption comprising leptin as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents.

Preferably, the composition inhibits, reduces or otherwise delays onset or progression of osteoclastogenesis.

The preferred form of a composition is as a pharmaceutical composition.

Compositions suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. They are generally stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyoil (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

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Sterile injectable solutions are prepared by incorporating leptin as hereinbefore defined in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by, for example, filter sterilization by other appropriate means. In the case of sterile powders for the preparation of sterile injectable solutions, a preferred method of preparation includes vacuum drying and freeze-drying which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution.

When leptin is suitable protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; and a lubricant such as magnesium stearate. Any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amount employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

Effective amounts of leptin will vary depending on the condition to be treated by may range from 0.001 ng/kg body weight to 100 mg/kg body weight. Leptin may be

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administered every minute or hourly, daily, weekly or monthly. Leptin may be used prophylactically or in the treatment of a disease condition.

5 The mode of administration may be intravenous, drip, infusion, oral, intraperitoneal, intra-bone, parenteral, inhalation, nasal drip, aerosol or rectal.

Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

10

The present invention further contemplates genetic modulation of endogenous leptin levels to thereby induce modulation of osteoclastogenesis.

15

Although not intending to limit the present invention to any one theory or mode of action, it is proposed herein that leptin inhibits osteoclastogenesis by antagonism of the osteoclastic effect of ODF by stimulation of OPG and inhibition of RANK expression.

20

The preferred subject for treatment is a human. The invention extends, however, to treatment in non-human animals such as primates, livestock animals (e.g. sheep, cows, pigs, goats, donkeys, horses), laboratory test animals (e.g. mice, rats, guinea pigs, hamsters), companion animals (e.g. dogs, cats) and captive wild animals.

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**EXAMPLE 1*****Human Peripheral Blood Mononuclear Cell (PBMC) Cultures***

PBMC were isolated from the peripheral blood of haemochromatic patients and healthy  
5 volunteers. Whole blood was spun at 700 g and serum discarded. Blood cells were then  
diluted 1:1 in PBS and layered over ficoll at a volume ratio of 5:3 and spun at 400 g for  
30 min. The top layer was discarded and the underlying layer containing the peripheral  
blood mononuclear cells (PBMCs) collected. PBMCs were washed in PBS to remove  
ficoll, collected *via* centrifugation (140 g) and resuspended in eagle's MEM/10% v/v  
10 FCS. PBMCs were seeded into 25 cm<sup>3</sup> tissue flasks (20-25 x 10<sup>6</sup> PBMCs/flask) and 4 x 4  
mm cortical bovine bone slices (1 x 10<sup>6</sup> PBMCs/bone slice) and left to adhere for 2 hrs.  
Flasks and bone slices were rinsed to remove non-adherent cells and fresh media added.

**EXAMPLE 2*****Action of Leptin***

The inventors proposed that systemic and/or bone marrow-derived leptin acted on  
osteoclast precursors (directly and/or indirectly) and regulated osteoclastogenesis. The  
inventors cultured adherent human peripheral blood monocytes (hPBMCs) on bone slices  
20 for 21 days in the presence of ODF and M-CSF (Quinn, 1998) with and without added  
leptin at various concentrations. The formation of multinuclear (MNC) osteoclasts was  
quantified by cytochemical staining for tartrate-resistant acid phosphatase (TRAP), <sup>125</sup>I-  
salmon calcitonin (<sup>125</sup>I-sCT) autoradiography for calcitonin receptor (CTR) and the  
identification of bone resorption lacunae by SEM.

The inventors found that leptin was a potent inhibitor of ODF-dependent osteoclast  
(TRAP+ve, CTR+ve MNCs) formation (Figure 1 and Table 3) and that this effect was  
dose-dependent (Figure 2). Consistent with this, was a corresponding dose-dependent  
decrease in bone resorption (Figure 3). At the highest concentration used (1.6 µg/ml),  
30 leptin reduced the number of osteoclasts and plan area of bone resorption by  
approximately 80%.

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Although not intending to limit the present invention to any one theory or mode of action, the inventors postulated that the mechanism of the antagonistic effect of leptin on ODF-induced osteoclast generation might be related to stimulation of ODF's decoy receptor, OPG and/or inhibition of its target cell receptor, RANK. To test this hypothesis, PBMCs were treated with increasing concentrations of leptin for 24 hrs and OPG and RANK mRNA expression assessed by RT-PCR. It was found that leptin (0.032-3.2 µg/ml) increased OPG mRNA and decreased RANK mRNA in a dose-dependent manner (Figure 4). The OPG and RANK PCR products were confirmed by restriction enzyme digest. (The OPG response will be confirmed by RNase protection assay and/or "real-time" PCR).

The inhibitory effect of leptin on osteoclast generation is also seen in co-cultures of osteoblast-like UMR 106-01 cells and PBMCs, which do not require the addition of ODF since it is produced by the UMR 106-01. Treatment of a number of osteoblast (rat calvarial, UMR 106-01, SAOs-2) and stromal (ST2, giant cell tumour, M3T3-L1) cells with leptin had no consistent effect on expression of OPG, ODF or RANK mRNA.

**TABLE 3** Effect of leptin on the generation of calcitonin receptor positive (CTR +ve) cells

Adherent PBMC's cultured on bone slices for 3 weeks in MEM/MCSF (25 ng/ml) ± ODF (30 ng/ml) ± Leptin (1.6 µg/ml). Bone slices reacted for TRAP activity and calcitonin binding determined autoradiography.

| Treatment       | MNC, CTR+ve | Mono       | Mono, CTR+ve |
|-----------------|-------------|------------|--------------|
| MCSF alone      | 0           | 1345 ± 270 | 0            |
| MCSF+ODF        | 367 ± 50    | 55 ± 9     | 16 ± 4       |
| MCSF+ODF+Leptin | 0           | 1380 ± 360 | 9 ± 3        |



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**EXAMPLE 3*****Gene Expression by Semi-Quantitative RT-PCR***

Cultured cells were directly lysed in RNeasy Lysis Buffer and total RNA extracted according to the manufacturer's instructions. For RT and PCR reactions, a Perkin Elmer/Cetus DNA Thermal Cycler was used. Reverse transcription was performed in the presence of 5 mM MgCl<sub>2</sub>, 1 mM deoxynucleotide mix, 3.2 mg random primers, 50 units RNase inhibitor and 20 units AMV reverse transcriptase. The final mixture was reacted at 25°C for 10 min, 42°C for 60 min and 95°C for 5 min to denature the enzyme.

Sense and antisense primers were designed using the MacVector program and synthesised by Gibco BRL, (Gaithersburg, MD). Sequences and sizes are defined in Table 4.

**TABLE 4**

| Gene  | Forward Primer                | Backward Primer              | Product Size |
|-------|-------------------------------|------------------------------|--------------|
| GAPDH | 5= CAGTCAGCCGCATCTTCTTTTG 3=  | 5= TGGTTCACACCCATGACGAAC 3=  | 464 bp       |
| OPG   | 5= GTACGTCAAGCAGGAGTGCAATC 3= | 5= TTCTTGAGCTGTGTTGCCG 3=    | 472 bp       |
| RANK  | 5= TTAAGCCAGTGCTTCACGGG 3=    | 5= ACGTAGACCACGATGATGTCGC 3= | 497 bp       |

PCR products were confirmed by restriction enzyme digest and all primer pairs spanned intron-exon splice sites allowing for the detection of mRNA only.

PCR amplification was performed with cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min. The reaction mixture contained 40 pmol of each primer, 200 mM dNTPs, 2 ml of 10X reaction buffer, optimised concentrations of MgCl<sub>2</sub>; 0.75 mM (OPG), and 1.0 mM (GAPDH and Rank), 1U Taq DNA polymerase, and sterile distilled water up to 20 ml. The mixture was then overlaid with paraffin oil. For semi-quantitative RT-PCR analysis, the optimal number

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of cycles for each gene was determined as follows: GAPDH, 20 cycles, OPG, 32 cycles and Rank, 30 cycles. PCR products were resolved on a 1.2% w/v agarose gel and visualised using ethidium bromide. The size of the bands were confirmed by a 100 bp DNA ladder (Gibco BRL, Gaithersburg, MD). Complementary DNA from a sample of human giant cell tumour was used a positive control as we found it to express all the genes studied. Band intensities were measured on the Kodak Digital Science™ 1D Image Analysis Software and expressed as a ratio of GAPDH intensity.

#### EXAMPLE 4

##### 10 *Osteoclastogenesis assays employing purified CD14+ cells*

Leptin treatment does not significantly inhibit osteoclastogenesis in assays that use highly purified CD14+ve cells cultured on bone slices for 21 days in the presence of ODF and M-CSF. CD14+ve cells were positively-selected from PBMCs using anti-CD14 antibody-labelled immunomagnetic beads and the VarioMACs system. Purity (90-95%) was confirmed with FACs analysis. CD14+ cells are highly efficient in the production of osteoclasts. PBMC populations depleted of CD14+ve cells (i.e. CD14-ve) are not able to generate osteoclasts in this assay (Figure 5).

20 Furthermore, leptin does not upregulate expression of OPG mRNA in purified CD14+ve cells, although down-regulation of RANK mRNA is observed.

These results indicate that the mechanism of leptin-induced inhibition of osteoclastogenesis is not *via* a direct effect of leptin on CD14+ cells, which appear to be the predominant adherent osteoclast precursor present in the PBMC fraction.

Thus, leptin appears to be acting via another cell type(s) present in the PBMC fraction. At this time the identity of this cell(s) is unknown.

30 The proposed mechanism of inhibition of osteoclast generation by leptin is shown in Figure 6.

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Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The  
5 invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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**CLAIMS**

1. A method of modulating bone resorption in a human or animal, said method comprising administering to said human or animal an effective amount of leptin or a derivative, homologue, analogue, chemical equivalent, antagonist or agonist thereof for a time and under conditions sufficient for the modulation of osteoclastogenesis.
2. A method according to Claim 1 wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in <400>7 after optimal alignment.
3. A method according to Claim 1 wherein the leptin or its derivative, homologue, antagonist or agonist is encoded by the nucleotide sequence set forth in <400>8 or a nucleotide sequence having at least 60% similarity to <400>8 after optimal alignment or a nucleotide sequence capable of hybridizing to <400>8 or its complementary form under low stringency conditions at 42°C.
4. A method according to Claim 1 or 2 or 3 wherein the modulation comprises a reduction in bone resorption.
5. A method according to Claim 4 for the treatment of osteoporosis or Paget's disease.
6. A method for inhibiting, reducing or otherwise delaying onset or progression of bone resorption in a human or animal, said method comprising administering to said human or animal an effective amount of a leptin or a derivative, homologue, analogue, chemical equivalent or agonist thereof for a time and under conditions sufficient to inhibit, reduce or otherwise delay onset or progression of osteoclastogenesis.

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7. A method according to Claim 6 wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in <400>7 after optimal alignment.
8. A method according to Claim 7 wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in <400>7 after optimal alignment.
9. A method according to Claim 6 or 7 or 8 for the treatment of osteoporosis or Paget's disease.
10. A composition comprising leptin or a derivative, homologue, analogue, chemical equivalent, antagonist or agonist thereof and one or more pharmaceutically acceptable carriers and/or diluents when used for modulating bone resorption.
11. A composition according to Claim 10 when used for inhibiting bone resorption.
12. A composition according to Claim 11 when used for the treatment of osteoporosis or Paget's disease.
13. A method for inhibiting osteoclastogenesis in a human or animal, said method comprising administering to said human or animal an amount of leptin or a derivative, homologue, analogue, chemical equivalent or agonist thereof effective to antagonize the osteoclastic effect of ODF by stimulation of OPG and/or inhibition of RANK expression.
14. A method according to Claim 13 wherein the leptin or its derivative, homologue, antagonist or agonist comprises an amino acid sequence having at least 60% similarity to the amino acid sequence set forth in <400>7 after optimal alignment.

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15. A method according to Claim 13 wherein the leptin or its derivative, homologue, antagonist or agonist is encoded by the nucleotide sequence set forth in <400>8 or a nucleotide sequence having at least 60% similarity to <400>8 after optimal alignment or a nucleotide sequence capable of hybridizing to <400>8 or its complementary form under low stringency conditions at 42°C.

16. A method according to Claim 13 or 14 or 15 for the treatment of osteoporosis or Paget's disease.

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**ABSTRACT**

The present invention relates generally to a method of modulating bone resorption and to agents useful for same. More particularly, the present invention provides for the use of leptin and its derivatives, homologues, analogues, antagonists or agonists to modulate osteoclastogenesis. Even more particularly, the present invention contemplates the treatment of disorders characterised by or associated with excessive bone resorption such as but not limited to osteoporosis and Paget's disease. The present invention further provides for the use of leptin and its derivatives, homologues, analogues, antagonists and agonists in the manufacture of a medicament for the modulation of bone resorption.

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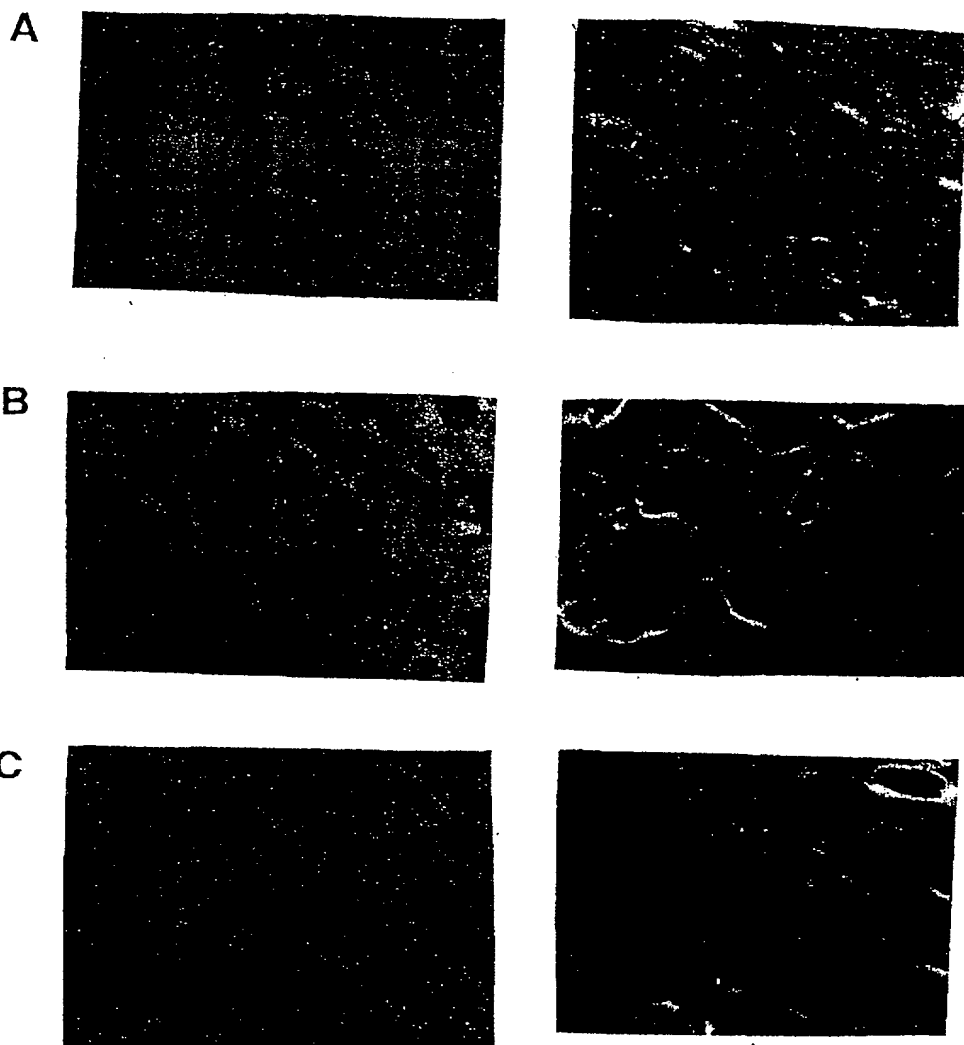


Figure 1

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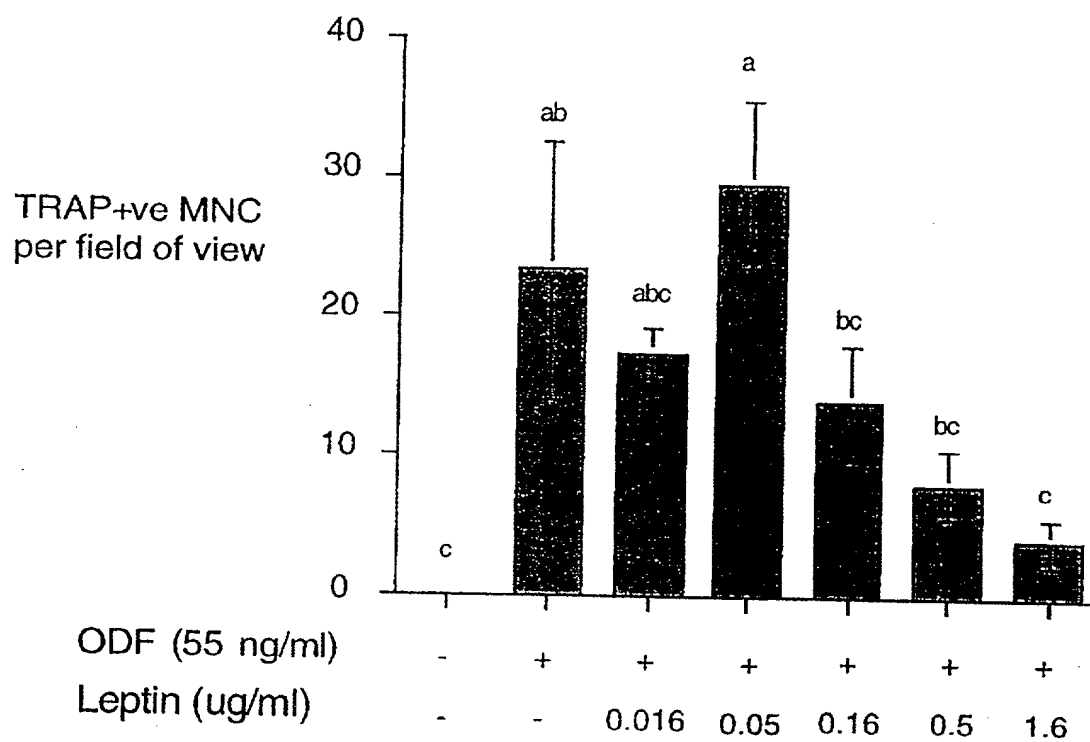


Figure 2

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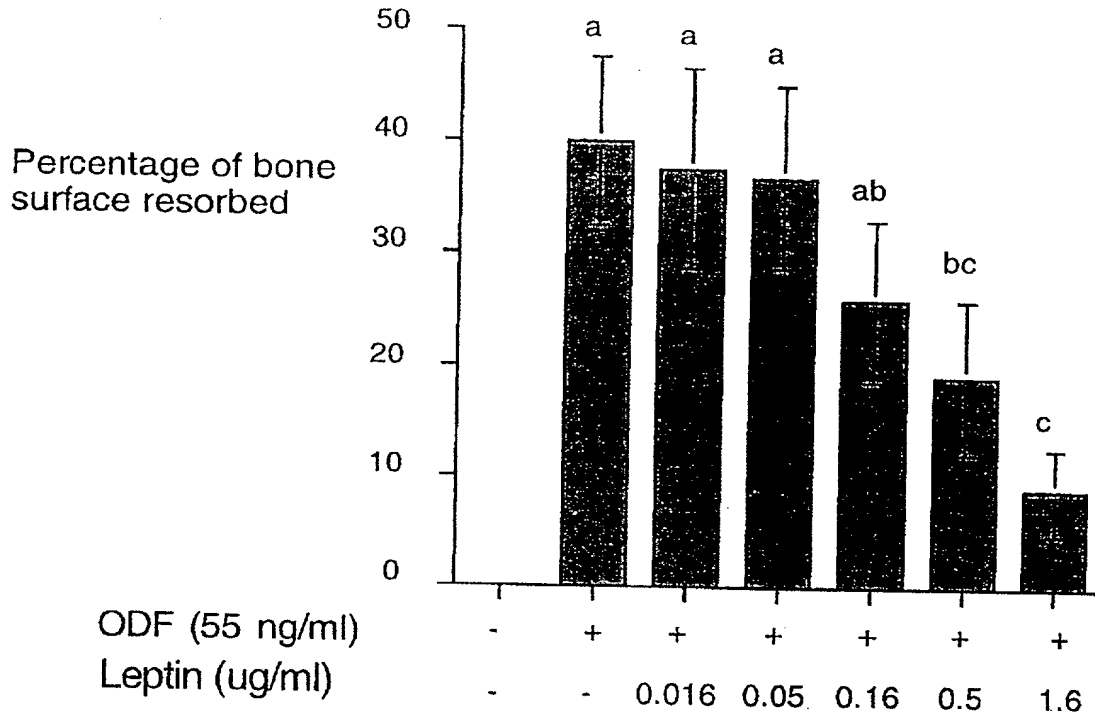
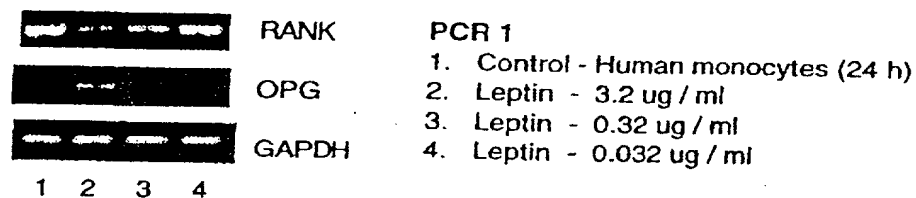
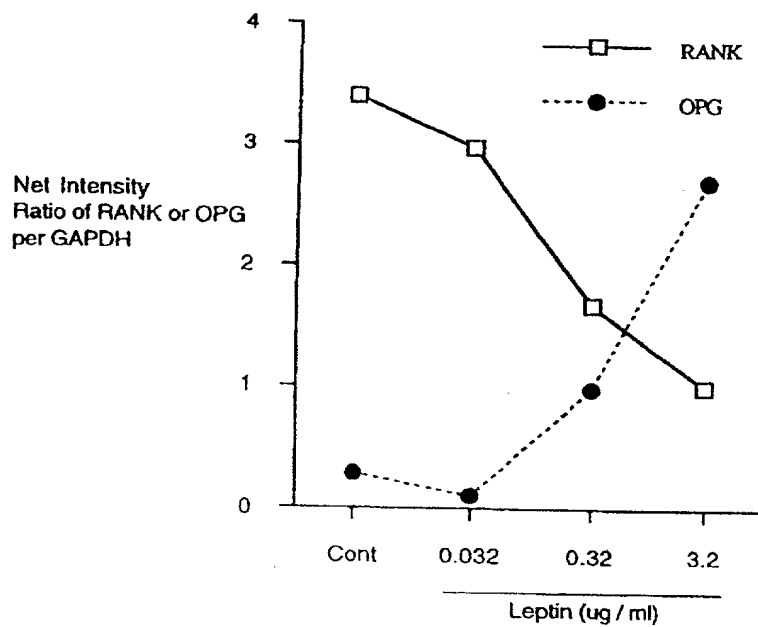


Figure 3

4/6

Human peripheral blood monocytes**Figure 4a**

## RANK and OPG expression in adherent PBMCs treated with leptin

**Figure 4b**

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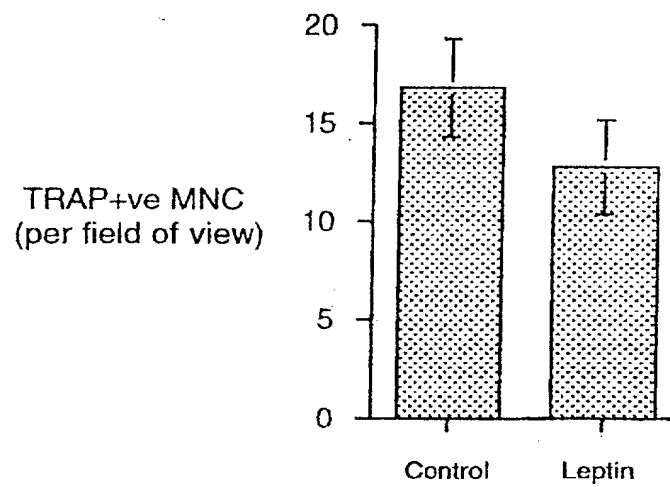


Figure 5a

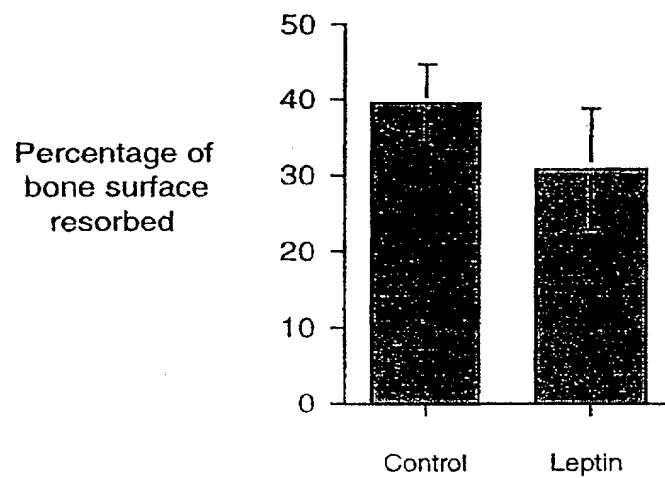


Figure 5b

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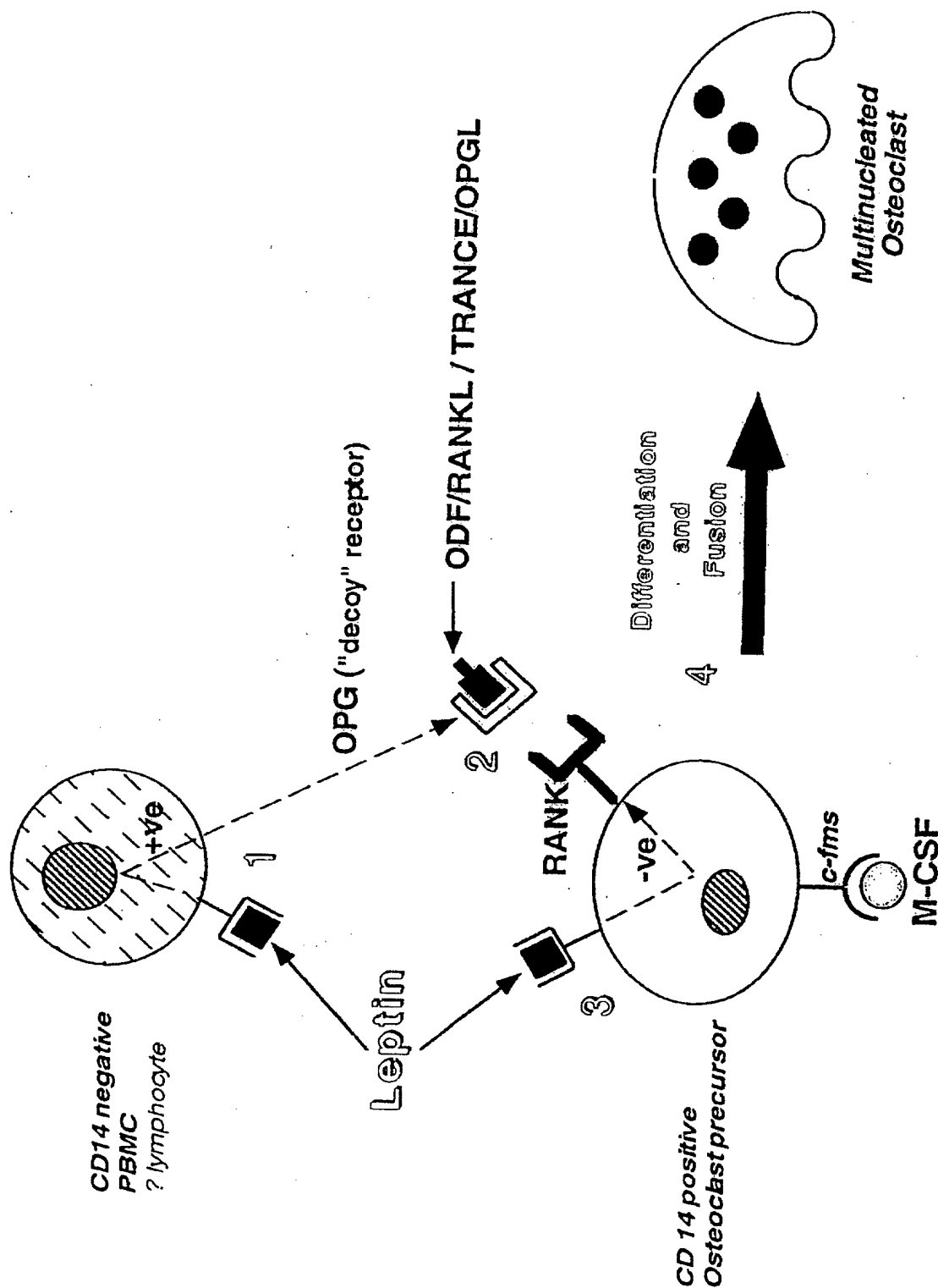


Figure 6

-i-

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&lt;210&gt; 1

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 1

ctactgcattc agatccaagg

20

&lt;210&gt; 2

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 2

gtcattggta ctggccaatc t

21

&lt;210&gt; 3

&lt;211&gt; 24

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- ii -

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 3

gcaatgcttt cactcctgag aaac

24

&lt;210&gt; 4

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 4

cagtaaacaac acagccacga caatgag

27

&lt;210&gt; 5

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 5

catggagaag gctggggctc

20

&lt;210&gt; 6

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 6

cactgacacg ttggcagtgg

20

&lt;210&gt; 7

&lt;211&gt; 504

&lt;212&gt; DNA

&lt;213&gt; Human cells

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- iii -

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1) .. (501)

&lt;400&gt; 7

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |    |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| atg | cat | tgg | gga | acc | ctg | tgc | gga | ttc | ttg | tgg | ctt | tgg | ccc | tat | ctt | 48 |
| Met | His | Trp | Gly | Thr | Leu | Cys | Gly | Phe | Leu | Trp | Leu | Trp | Pro | Tyr | Leu |    |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |    |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |    |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| ttc | tat | gtc | caa | gct | gtg | ccc | atc | caa | aaa | gtc | caa | gat | gac | acc | aaa | 96 |
| Phe | Tyr | Val | Gln | Ala | Val | Pro | Ile | Gln | Lys | Val | Gln | Asp | Asp | Thr | Lys |    |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     |     | 30  |     |    |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| acc | ctc | atc | aag | aca | att | gtc | acc | agg | atc | aat | gac | att | tca | cac | acg | 144 |
| Thr | Leu | Ile | Lys | Thr | Ile | Val | Thr | Arg | Ile | Asn | Asp | Ile | Ser | His | Thr |     |
|     |     |     | 35  |     |     |     |     | 40  |     |     |     |     |     | 45  |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| cag | tca | gtc | tcc | tcc | aaa | cag | aaa | gtc | acc | ggg | ttg | gac | ttc | att | cct | 192 |
| Gln | Ser | Val | Ser | Ser | Lys | Gln | Lys | Val | Thr | Gly | Leu | Asp | Phe | Ile | Pro |     |
|     |     |     | 50  |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ggg | ctc | cac | ccc | atc | ctg | acc | tta | tcc | aag | atg | gac | cag | aca | ctg | gca | 240 |
| Gly | Leu | His | Pro | Ile | Leu | Thr | Leu | Ser | Lys | Met | Asp | Gln | Thr | Leu | Ala |     |
|     | 65  |     |     |     |     |     | 70  |     |     |     | 75  |     |     |     | 80  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| gtc | tac | caa | cag | atc | ctc | acc | agt | atg | cct | tcc | aga | aac | gtg | atc | caa | 288 |
| Val | Tyr | Gln | Gln | Ile | Leu | Thr | Ser | Met | Pro | Ser | Arg | Asn | Val | Ile | Gln |     |
|     |     |     |     |     |     | 85  |     |     |     | 90  |     |     |     |     | 95  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ata | tcc | aac | gac | ctg | gag | aac | ctc | cgg | gat | ctt | ctt | cac | gtg | ctg | gcc | 336 |
| Ile | Ser | Asn | Asp | Leu | Glu | Asn | Leu | Arg | Asp | Leu | Leu | His | Val | Leu | Ala |     |
|     |     |     |     |     |     | 100 |     |     |     | 105 |     |     |     |     | 110 |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ttc | tct | aag | agc | tgc | cac | ttg | ccc | tgg | gcc | agt | ggc | ctg | gag | acc | ttg | 384 |
| Phe | Ser | Lys | Ser | Cys | His | Leu | Pro | Trp | Ala | Ser | Gly | Leu | Glu | Thr | Leu |     |
|     |     |     |     |     |     | 115 |     |     |     | 120 |     |     |     |     | 125 |     |

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- iv -

gac agc ctg ggg ggt gtc ctg gaa gct tca ggc tac tcc aca gag gtg 432  
 Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val  
 130 135 140

gtg gcc ctg agc agg ctg cag ggg tct ctg cag gac atg ctg tgg cag 480  
 Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln  
 145 150 155 160

ctg gac ctc agc cct ggg tgc tga 504  
 Leu Asp Leu Ser Pro Gly Cys  
 165

&lt;210&gt; 8

&lt;211&gt; 167

&lt;212&gt; PRT

&lt;213&gt; Human cells

&lt;400&gt; 8

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr Leu  
 1 5 10 15

Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys  
 20 25 30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr  
 35 40 45

Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro  
 50 55 60

Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala  
 65 70 75 80

Val Tyr Gln Gln Ile Leu Thr Ser Met Pro Ser Arg Asn Val Ile Gln  
 85 90 95

Ile Ser Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Val Leu Ala

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- V -

100

105

110

Phe Ser Lys Ser Cys His Leu Pro Trp Ala Ser Gly Leu Glu Thr Leu  
 115 120 125

Asp Ser Leu Gly Gly Val Leu Glu Ala Ser Gly Tyr Ser Thr Glu Val  
 130 135 140

Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Met Leu Trp Gln  
 145 150 155 160

Leu Asp Leu Ser Pro Gly Cys  
 165

&lt;210&gt; 9

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 9

cagtcagccg catcttcttt tg

22

&lt;210&gt; 10

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 10

tggttcacac ccatgacgaa c

21

&lt;210&gt; 11

&lt;211&gt; 23

&lt;212&gt; DNA

&lt;213&gt; Human cells

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- vi -

&lt;400&gt; 11

gtacgtcaag caggagtgc atc

23

&lt;210&gt; 12

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 12

ttcttgtgag ctgtgttgcc g

21

&lt;210&gt; 13

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 13

ttaagccagt gcttcacggg

20

&lt;210&gt; 14

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Human cells

&lt;400&gt; 14

acgtagacca cgatgatgtc gc

22

002020"4/02E960